PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
C12N 11/04, 11/06, C12M 3/00

A1

(11) International Publication Number: WO 91/07485

(43) International Publication Date: 30 May 1991 (30.05.91)

(21) International Application Number: PCT/US90/06554

(22) International Filing Date: 8 November 1990 (08.11.90)

(30) Priority data:
434,092 9 November 1989 (09.11.89) US

(71) Applicant: BIO-METRIC SYSTEMS, INC. [US/US]; 9924 West 74th Street, Eden Prairie, MN 55344 (US).

(72) Inventors: CLAPPER, David, L. ; 6110 Chestnut Terrace, Shorewood, MN (US). HU, Wei-Shou ; 1810 Howell Street, Falcon Heights, MN 55113 (US).

(74) Agents: BAUMAN, Mary, P. et al.; Fredrikson & Byron, 1100 International Centre, 900 2nd Avenue S., Minneapolis, MN 55402-3397 (US).

(81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent).

Published
With international search report.

(54) Title: IMPROVED BIOREACTOR SURFACES AND METHODS OF MAKING SAME

(57) Abstract

Improved bioreactor surfaces, and methods for their production and use, are disclosed herein. These improved bioreactor surfaces carry a composition of a cell adhesion factor and a positively charged moiety sufficient to improve cell attachment and to stabilize cell growth. Desirably, one or both of the cell adhesion factor and the positively charged chemical moiety is directly or indirectly covalently linked to the bioreactor surface. Alternately, the cell adhesion factor and the positively charge chemical moiety may be bound to one another and either the cell adhesion factor or the positively charged chemical moiety bound directly or indirectly to the supporting surface.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	**FI**	Finland	**ML**	Mali
AU	Australia	**FR**	France	**MN**	Mongolia
BB	Barbados	**GA**	Gabon	**MR**	Mauritania
BE	Belgium	**GB**	United Kingdom	**MW**	Malawi
BF	Burkina Faso	**GN**	Guinea	**NL**	Netherlands
BG	Bulgaria	**GR**	Greece	**NO**	Norway
BJ	Benin	**HU**	Hungary	**PL**	Poland
BR	Brazil	**IT**	Italy	**RO**	Romania
CA	Canada	**JP**	Japan	**SD**	Sudan
CF	Central African Republic	**KP**	Democratic People's Republic of Korea	**SE**	Sweden
CG	Congo			**SN**	Senegal
CH	Switzerland	**KR**	Republic of Korea	**SU**	Soviet Union
CI	Côte d'Ivoire	**LI**	Liechtenstein	**TD**	Chad
CM	Cameroon	**LK**	Sri Lanka	**TG**	Togo
DE	Germany	**LU**	Luxembourg	**US**	United States of America
DK	Denmark	**MC**	Monaco		
ES	Spain	**MG**	Madagascar		

IMPROVED BIOREACTOR SURFACES AND METHODS OF MAKING SAME

This invention was made with government support under grant number ISI-8760680 awarded by the National Science Foundation. The government has certain rights in the invention.

FIELD OF THE INVENTION

This invention relates to cell culture surfaces of bioreactors in the field of cell biology and particularly to methods of improving the surfaces to obtain better cell attachment and cell growth.

BACKGROUND OF THE INVENTION

Cell culture of mammalian cells has long been used for the production of many vaccines and genetically engineered proteins. Animal cells are generally categorized according to their anchorage-dependence. Some cell types, such as lymphocytes, can grow in suspension, others, called "anchorage-dependent", including fibroblasts and epithelial and endothelial cells, must attach to a surface and spread out in order to grow. Other cells can grow either in suspension or anchored to a surface.

Anchorage-dependent cells have historically been cultivated on the walls of roller bottles or non-agitated vessels such as tissue culture flasks, which are used in many laboratories. As the necessity has developed to provide large amounts of certain antiviral vaccines, genetically engineered proteins, and other cell-derived products, attempts have been made to develop new systems for larger scale production of cells.

The first focus of this development effort was to increase the growth surface area per unit vessel volume and to implement convenient and appropriate environmental controls. Some of these technologies involved the use of packed-glass beads, stacked plates, rotating multiple tubes, and roller bottles with spiral films inside.

Among the most important advances in the field of cell culture technology have been the use of microcarriers and more recently, the use of hollow fiber systems. Original microcarriers developed by van Wezel (van Wezel, A.L., "Growth of Cell-Strains and Primary Cells on Micro-carriers in Homogeneous Culture," Nature 216:64-65 (1967)) consisted of positively charged DEAE-dextran beads suspended in culture media in a stirred vessel. Cells would attach to the bead surface and grow as a monolayer.

Hollow fiber bioreactor configurations serve to compartmentalize the bioreactors. In one common configuration, these units allow cells to grow on the outside surfaces of bundles of parallel fibers enclosed in an outer compartment. Nutrient- and gas-enriched medium flows through the fibers' hollow centers. Cell products are concentrated in the outer compartment of the bioreactor because the inner surface of the fiber includes an ultrafiltration membrane that excludes large molecular-weight cell products.

Bioreactors have certain minimum requirements: an aeration system is required to bring the correct amount of oxygen to the cells without causing shear damage; surfaces are required for supporting anchorage-dependent cells; and means are required to enable operators to sample and monitor the contents of the bioreactor without contaminating the culture.

The various bioreactors have encountered similar problems in culturing cells. With respect to anchorage-dependent cells, specific cell culture parameters in need of improvement include: (1) better initial attachment and growth of cells to decrease the concentration of cells required for inoculation of a culture; (2) improved long-term cell adhesion, viability, and productivity to increase the useful life of the bioreactor; and (3) alteration of growth conditions to allow lower concentrations of serum to be used in the culture medium.

For many of the anchorage-dependent cell types, it is believed that the closer the surface composition is to that encountered by the cell in vivo, the better the cell plating efficiency, growth rate, maintenance of the differentiated state, and long-term viability. Cell growth has been greatly improved using collagen-based extracellular matrix preparations. However, the composition of extracellular matrix preparations is not well defined, and such preparations are expensive to produce and are susceptible to microbial contamination prior to use. Several cell adhesion proteins (e.g., fibronectin, laminin, and collagen) have been purified from extracellular matrix preparations and used on tissue culture surfaces to promote cell attachment and cell growth. Kleinman, H.K., L. Luckenbill-Edds, F.W. Cannon, and G.C. Sephel, "Use of Extracellular Matrix Components for Cell Culture," Anal. Biochem. 166:1-13 (1987) Studies have shown that a coating of gelatin or denatured collagen on microcarriers facilitates the attachment and growth of mammalian cells. Microcarrier Cell Culture. Principles and Methods, Pharmacia Fine Chemicals, Uppsala, Sweden, pages 5-33 (1981).

Early microcarriers were in the form of DEAE-derivatized dextran beads. The use of these beads, however, produced certain deleterious effects. For example, a high initial cell death rate and inadequate cell growth have been observed in cells attached to beads that contain an ion exchange capacity that is too high. One method that was proposed to overcome some of the deleterious effects involved attaching positively-charged molecules to the beads to provide a charge capacity of 0.1-4.5 meq/g dextran (e.g., U.S. Patent 4,293,654). U.S. Pat. 4,036,693 (Levine, et al) shows the treatment of microcarriers with commercially available ion exchange residues having macromolecular polyanions.

Cell adhesion proteins such as fibronectin, collagen and laminin have worked well to promote the growth and spreading of cells but have not attracted or attached cells to the surfaces with sufficient rate or tenacity. As a result, the efficiency of cell adhesion proteins has been limited by the manner in which such proteins can come into contact with cells. On the other hand, positively-charged moieties tend to increase initial cell attachment but have been reported to have a negative effect on growth and product yield. Tao, T-Y. et al., "Kinetics of Prourokinase Production by Human Kidney Cells in Culture," J. Biotechnol.6:205-224 (1987). Cells readily attach to a surface through the use of these positively-charged chemical moieties, but growth for some reason seems to be suboptimal. Too high a density of charged groups produces toxic effects and inhibits cell growth. Butler, M., "Growth Limitations in Microcarrier Cultures," Advances Biochem. Eng./Biotech 34:57-84 (1987); Microcarrier Cell Culture. Principles and Methods, Pharmacia Fine

Chemicals, Uppsala, Sweden (1981); Tao, T.Y., G.Y. Ji, and W.S. Hu, "Human Fibroblastic Cells Attach to Controlled-Charge and Gelatin-Coated Microcarriers at Different Rates," J. Biotechnol. 6:9-12 (1987).

SUMMARY OF INVENTION

It has been discovered that bioreactor cell culture surfaces can be improved to increase the rate of cell attachment without impairing cell growth on the surface by providing the surface with a composition comprising a positively charged moiety or component and a cell adhesion factor.

In one embodiment, the composition comprises a cell adhesion factor and a positively charged moiety each separately covalently bound to the supporting surface. Desirably, one or both of the cell adhesion factor and the positively charged chemical moiety is covalently linked to the said surface through a linking group, the linking group including the residue of a latent reactive group employed to covalently bond to the supporting surface. Alternately, the cell adhesion factor and the positively charged chemical moiety may be bound to one another and either the cell adhesion factor or the positively charged chemical moiety bound to the supporting surface.

In another embodiment, the cell adhesion factor and the positively charged moiety of the composition each may be adhered to the supporting surface, or they may be bound to one another and either the cell adhesion factor or the positively charged chemical moiety adhered to the supporting surface. In any event, the cell adhesion factor and positively charged moiety each are uniformly and homogeneously distributed on the surface and together form an homogeneous aggregate or blend of these materials on the surface and they are located with respect to each

other on the surface so that both are presented to cells to be affixed to the surface. The supporting surface of the bioreactor bears a sufficient density of a cell adhesion factor and a sufficient density of a positively charged moiety to promote and stabilize cell attachment to the surface.

Yet another embodiment of the invention comprises a method of growing anchorage-dependent cells on a supporting surface of a cell culture system comprising attaching to the supporting surface a positively-charged moiety and a cell adhesion factor to form a homogeneous composition on the surface, and combining anchorage-dependent cells with the supporting surface in an aqueous environment.

DETAILED DESCRIPTION OF THE INVENTION

A number of bioreactor configurations, as described above, exist for culturing anchorage-dependent cells, and the invention is not dependent upon any particular configuration. The bioreactor surface of this invention comprises a supporting cell culture surface bearing a composition comprising a positively charged moiety and a cell adhesion factor. The supporting surface is desirably water insoluble. For example, the supporting surface may be a high molecular weight polymer, such as polysaccharides (e.g., dextran, dextrin, starch or cellulose), polystyrene, polyvinyl alcohol, acrylate or methacrylate polymers, and glass. Preferably, the supporting surface is a microcarrier bead comprising either DEAE-substituted dextran, dextran coated with gelatin, polyacrylamide, polystyrene, porous glass, cellulose, or fluorocarbon droplets.

For bioreactors that utilize microcarriers, the size of the microcarrier bead used will depend upon the cell type being cultivated. Larger beads minimize

the required concentration of cells needed to inoculate a culture and maximize the growth rate, whereas small beads maximize the surface area and require less vigorous mixing to remain in suspension. Also, different cell types grow optimally on different sizes of microcarriers.

This invention improves the attachment and growth of anchorage-dependent cells on bioreactor surfaces. Such cells must attach to a surface and spread out in order to grow. Grinnel, F., "Cellular Adhesiveness and Extracellular Substrata," International Rev. Cytology 53:65-114 (1978). Two types of commonly used anchorage-dependent cells are green monkey kidney (Vero) cells and Chinese hamster ovary (CHO) cells. Cell attachment to bioreactor surfaces can be either receptor-mediated or not mediated by cell receptors. With receptor-mediated cell attachment, receptors on the cell surface recognize and bind to cell adhesion factors carried by the bioreactor surfaces. Yamada, K.M., "Cell Surface Interactions with Extracellular Materials," Ann. Rev. Biochem. 52:761-799 (1983). Kleinman, H.K., L. Luckenbill-Edds, F.W. Cannon, and G.C. Sephel, "Use of Extracellular Matrix Components for Cell Culture," Anal. Biochem. 166:1-13 (1987). Cell adhesion factors immobilized onto bioreactor surfaces are believed to promote receptor-mediated cell attachment.

A second type of cell attachment is not mediated by cell surface receptors. The positively-charged moieties employed on bioreactor surfaces of the invention may promote cell attachment to by promoting ionic binding between the positively-charged moieties and e.g. negatively-charged glycoproteins and phospholipids carried on cell surfaces. By combining cell adhesion factors and positively-charged moieties

on the same bioreactor surface, this invention produces superior cell attachment by a mechanism which we theorize involves both receptor-mediated and non-receptor- mediated cell attachment.

"Cell adhesion factors" are molecules that include cell adhesion proteins, cell adhesion protein peptide fragments, synthetic peptide analogs and the like which mediate the adherence of cells, via the cell's receptors, to a surface and which increase the rate at which such cells grow and spread on that surface. Cell adhesion factors useful with this invention may include such cell adhesion proteins as laminin, fibronectin, collagens (all types), vitronectin, and tenascin, such cell adhesion peptides as the cell attachment domain of fibronectin identified as the tripeptide (RGD) and the cell attachment domain of laminin identified as the pentapeptide (YIGSR) of laminin, as well as other binding domains of these and other cell adhesion proteins and functional synthetic analogs thereof.

Cell adhesion proteins have one or more domains that mediate binding to cell surface receptors. These cell attachment domains consist of specific amino acid sequences that can be chemically synthesized to produce cell adhesion peptides that possess the cell attachment properties of the intact cell adhesion proteins. Two examples of such cell adhesion peptides are the tripeptide (RGD or arg-gly-asp) sequence present in fibronectin and the pentapeptide (YIGSR or tyr-ile-gly-ser-arg) sequence present in laminin. Ruoslahti, E. and M. Pierschbacher "Arg-Gly-Asp: A versatile Cell Recognition Signal," Cell 44:517-518 (1986). Pierschbacher, M.D. and E. Ruoslahti, "Cell Attachment Activity of Fibronectin can be Duplicated by Small Synthetic Fragments of the Molecule," Nature

309:30-33 (1984). Graf, J. et al., "Identification of an Amino Acid Sequence in Laminin Mediated Cell Attachment, Chemotaxis, and Receptor Binding," Cell 48:989-996 (1987).

Cell adhesion proteins are primarily those that are naturally occurring and quite large, with molecular weights above about 100,000 daltons. Cell adhesion peptides generally are short amino acid sequences derived from or functionally analogous to the binding domains of the cell adhesion proteins. Desirably, cell adhesion peptides used in this invention have between about 3 and 30 amino acid moieties in their amino acid sequences. Preferably, cell adhesion peptides have not more than about 15 amino acid moieties in their amino acid sequences.

The density of of cell adhesion factor and positively charged moiety that is sufficient to promote initial cell attachment and to stabilize attachment of the cells to the surface will vary and will depend in part upon such factors as the configuration of the bioreactor, the material with which the supporting surface is made, and the cells to be attached to the surface. A sufficient density of cell adhesion factor should be carried by the bioreactor's supporting surface to promote cell attachment and growth. For example, the density of cell adhesion factor will desirably range from about 0.1 nanomole to about 10 micromoles of factor per gram of dextran or non-porous polystyrene. With cell adhesion proteins, the desirable range is from 0.1 nanomole to 10 nanomoles, and with the cell adhesion peptides, the desirable range is from 10 nanomoles to 10 micromoles per gm of dextran or non-porous polystyrene.

Positively charged moieties useful with this invention include such tertiary amine and quaternary ammonium groups as are described, for example, in U.S. Patent No. 4,189,534 (Levine, et al), the disclosure of which is incorporated herein by reference. Particularly desired materials of this type are substituted amines or salts thereof, including trialkyl- substituted, tetra-alkyl substituted or mono- or di-substituted alkyl amines. The tetraalkyl amines, of course, are quaternary ammonium salts. Other positively-charged moieties include diethylaminoethylchloride, diethylaminoethylbromide, dimethylaminoethylchloride, dimethylaminoethylbromide, di-(hydroxyethyl)-aminoethylchloride, di-(hydroxyethyl)-aminoethylbromide, di-(hydroxyethyl)-aminomethylchloride, di-(hydroxyethyl)- aminomethylbromide, ↑-morpholinoethylchloride, ↑-morpholinoethylbromide, ↑-morpholinomethylchloride, ↑-morpholinomethylbromide and salts thereof, for example, the hydrochlorides.

Particularly preferred moieties used to increase the positive charge density of the supporting surface are dimethyl-aminopropylamine (DMAPA), chitosan, and similar poly(cationic amino acids) such as polyarginine and polyornithine, and polylysine.

The density of the positively charged moiety sufficient to promote initial cell attachment and to stabilize attachment of the cells to the surface depends upon such factors as the configuration of bioreactor used, the material from which the supporting surface is made and the cells that are to be attached to the surface. A sufficient density of positively charged moiety should be carried by the bioreactor's supporting surface to provide the surface with an ion exchange capacity at a physiologic pH to

promote initial cell attachment. Ion exchange capacity is a quantitative measurement of the amount of a negatively charged reagent that can bind to the bioreactor surface. The rate of cell attachment to a bioreactor surface is reported to correlate more closely with ion exchange capacity than with charge density. Himes, V.B. and W. S. Hu, "Attachment and Growth of Mammalion Cells on Microcarriers with Different Ion Exchange Capacities," Biotechnol. Bioeng. 29:1155-1163 (1987). Desirably, the density of positively charged moiety in this invention is such as to provide the bioreactor surface with an ion exchange capacity in the range of 0.2 to 2.0 meq/gm for dextran and 0.02 to 0.2 meg/gm for non-porous polystyrene.

The exchange capacity of a DEAE-dextran bead microcarrier supporting surface carrying a positively charged moiety can be determined by titration of bound DEAE-HCl molecules as described by Levine, et al, Biotechnol. Bioeng. Vol. 21, 821 (1979), the disclosure of which is incorporated herein by reference. The DEAE-dextran microcarriers are typically washed with 0.1 molar HCl (0.5 L/g dry dextran beads) to allow for the saturation of the exchange sites with chloride ions. In order to remove unbound chloride ions, the beads are rinsed with dilute HCl (10^{-4} M, 0.8 L/g dry dextran beads). The beads are then washed with 10% (w/w) sodium sulfate (75 mL/g dry dextran beads) and the filtrate collected. The last wash displaces the bound chloride ions with sulfate ions. 100 ml of the filtrate is titrated with 1.0 molar silver nitrate in the presence of potassium chromate as an indicator (1 mL 5% w/w solution).

The cell adhesion factors and positively charged moieties may be attached directly or indirectly through a linking moiety to the supporting surface through any suitable attachment means, such as adsorption, ionic or covalent binding and the like.

Certain strongly adsorbing reagents may be used to immobilize the cell attachment factors and positively charged moieties onto cell culture surfaces. One class of such strongly adsorbing reagents is cationic surfactants. Two examples of cationic surfactants are benzalkonium chloride and tridodecylmethylammonium chloride (TDMAC). The hydrophobic portions of the surfactant molecules adhere strongly to hydrophobic surfaces (e.g., polystyrene), thus immobilizing the positively charged e.g., quaternary amine portions. In the present invention, the hydrophobic portions of a surfactant molecule may be covalently bonded to cell attachment factors and/or positively charged moieties prior to adsorption onto the cell culture surface.

Different types of cell adhesion factors, such as gelatin and one or more cell attachment peptides, may be carried upon the same supporting surface. Cell adhesion factors promote cell attachment by binding to specific receptors on the cell surface, and some cell types have receptors for more than one type of cell adhesion factor. Buck, C.A. and A.F. Horwitz, "Cell Surface Receptors for Extracellular Matrix Molecules," Ann. Rev. Cell Biol. 3:179- 205 (1987). Immobilizing different types of cell adhesion factors upon the same supporting surface may allow the binding to more receptors on each cell than would occur with a simple type of immobilized cell adhesion factor, therefore possibly resulting in faster and more tenacious cell attachment to the supporting surface.

Preferably, cell adhesion factors and positively charged moieties are each covalently bound to the supporting surface. Desirably, one or both of the cell adhesion factor and the positively charged chemical moiety is covalently linked to the said surface through a linking group, the linking group including the residue of a latent reactive group through which the cell adhesion factor or positively charged moiety is covalently bonded to the supporting surface. We have discovered that covalent bonding to the supporting surface of a cell adhesion factor that has attached to it a positively charged moiety leads to faster cell attachment than occurs to supporting surfaces that have attached only a cell adhesion factor or a positively charged moiety.

Latent reactive groups, broadly defined, are groups which respond to specific applied external stimuli to undergo active species generation with resultant covalent bonding to an adjacent support surface. Latent reactive groups are those groups of atoms in a molecule which retain their covalent bond unchanged under conditions of storage but which, upon activation, form covalent bonds with other molecules. The latent reactive groups generate active species such as free radicals, nitrenes, carbenes, and excited states of ketones upon absorption of external electromagnetic or kinetic (thermal) energy. Latent reactive groups may be chosen to be responsive to various portions of the electromagnetic spectrum, and latent reactive groups that are responsive to ultraviolet, visible or infrared portions of the spectrum are preferred. Latent reactive groups as described are generally well known.

The azides constitute a preferred class of latent reactive groups and include arylazides ($C_6R_5N_3$)

such as phenyl azide and particularly 4-fluoro-3-nitrophenyl azide, acyl azides ($-CO-N_3$) such as benzoyl azide and p-methylbenzoyl azide, azido formates ($-O-CO-N_3$) such as ethyl azidoformate, phenyl azidoformate, sulfonyl azides ($-SO_2-N_3$) such as benzenesulfonyl azide, and phosphoryl azides $(RO)_2PO-N_3$ such as diphenyl phosphoryl azide and diethyl phosphoryl azide. Diazo compounds constitute another class of latent reactive groups and include diazoalkanes ($-CHN_2$) such as diazomethane and diphenyldiazomethane, diazoketones ($-CO-CHN_2$) such as diazoacetophenone and 1-trifluoromethyl-1-diazo-2-pentanone, diazoacetates ($-O-CO-CHN_2$) such as t-butyl diazoacetate and phenyl diazoacetate, and beta-keto-alpha- diazoacetates ($-CO-CHN_2-CO-O-$) such as t-butyl alpha diazoacetoacetate. Other latent reactive groups include the aliphatic azo compounds such as azo-bis-isobutyronitrile, the diazirines ($-CHN_2$) such as 3-trifluoromethyl-3-phenyldiazirine, the ketenes ($-CH=C=O$) such as ketene and diphenylketene and photoactivatable ketones such as benzophenone and acetophenone. Peroxy compounds are contemplated as another class of latent reactive groups and include dialkyl peroxides such as di-t-butyl peroxide and dicyclohexyl peroxide and diacyl peroxides such as dibenzoyl peroxide and diacetyl peroxide and peroxyesters such as ethyl peroxybenzoate.

Upon activation of the latent reactive groups to cause covalent bond formation to the surfaces to which polymer molecules are to be attached, the polymer molecules are covalently attached to the surfaces by means of residues of the latent reactive groups. Exemplary latent reactive groups, and their residues upon activation, are as follows:

Latent Reactive Group	Residue Functionality	
aryl azides	amine	R-NH-R'
acyl azides	amide	R-CO-NH-R'
azidoformates	carbamate	R-O-CO-NH-R'
sulfonyl azides	sulfonamide	$R-SO_2-NH-R'$
phosphoryl azides	phosphoramide	$(RO)_2PO-NH-R'$
diazoalkanes	new C-C bond	
diazoketones	new C-C bond & ketone	
diazoacetates	new C-C bond & ester	
beta-keto-alpha-diazoacetates	new C-C bond & B-ketoester	
aliphatic azo	new C-C bond	
diazirines	new C-C bond	
ketenes	new C-C bond	
photoactivated ketones	new C-C bond & alcohol	
dialkyl peroxides	ethers	
diacyl peroxides	esters & new C-C bonds	
peroxyesters	ethers, esters, and new C-C bonds	

The supporting surface of the bioreactor of this invention desirably has a mole ratio of positively charged chemical moiety to the cell adhesion factor in the range of about 10:1 to about 10^6:1. It is to be understood that the mole ratio will vary according to the size of cell adhesion factor used. For example, if the cell adhesion factor is an intact cell adhesion protein the mole ratio of positively charged chemical moiety to the cell adhesion factor is desirably in the range of about 10^4:1 to about 10^6:1.

The reason that the presence of both a cell adhesion factor and a positively charged chemical moiety on a bioreactor surface result in better initial cell attraction and better attachment and growth of cells to bioreactor surfaces is not

thoroughly understood. While not wishing to be bound by this theory, we think it likely that positively charged chemical moieties attract and cause initial attachment of cells to the bioreactor surfaces, thus bringing the cells into functional proximity with the cell adhesion factors which then bind the cells more firmly and promote cell spreading.

In bioreactor systems used for large-scale production of cells, the culture media must be mixed or perfused to replenish nutrients and to remove waste products from cells. The shear forces that result from the culture medium passing over cell culture surfaces interfere with the attachment of cells. These shear forces are usually greatest in stirred microcarrier bioreactors, less in roller bottles, and least in hollow fiber bioreactors. However, in each of these bioreactor systems, certain commercially important cell lines do not attach well. The present invention may be employed to improve cell attachment and growth in all of these bioreactor systems.

Since the invention improves cell attachment in the presence of disruption shear forces, the immobilized cell adhesion factors and positively-charged moieties must be tenaciously bound to the culture surface to firmly anchor the attached cells. Covalent immobilization is the preferable method for providing such tenacious immobilization of the cell adhesion factors and positively-charged moieties. Results presented in Example 8 demonstrate that cells bind faster and more tenaciously to covalently-immobilized cell adhesion proteins and positively-charged moieties than to the same reagents when they are adsorbed.

The invention is further illustrated by the following non-limiting examples:

Example 1: Preparation of Microcarriers and Reagents

Microcarrier Beads. Microcarrier beads suspended in spinner flasks and rotated vials were used as bioreactor configurations, since the beads can be readily removed at timed intervals to determine the density of attached cells. Two types of microcarrier beads were used for surface modification: gelatin-coated dextran (Cytodex 3 from Pharmacia Fine Chemicals, Uppsala, Sweden) and polystyrene (100 micrometer diameter polystyrene/divinyl benzene beads from Seradyn, Inc., Indianapolis, IN). Dextran is currently the most widely used microcarrier material but has the disadvantage of being both porous and compressible; both are properties that complicate the harvesting of products secreted into the media. Polystyrene was used as a non-compressible alternate.

Polystyrene beads with an average diameter of 100 micrometers were used. Cell attachment and growth on Cytodex 1 and Cytodex 3 beads (both from Pharmacia) with controlled charge and immobilized gelatin, respectively, were assayed as controls for commercially available surface-modified beads. The Cytodex beads have respective average diameters of 180 and 175 micrometers.

Reagents. Denatured (acid cured) porcine skin gelatin (Type A, 300 bloom from Sigma) was used for immobilization onto polystyrene beads. This gelatin is composed mostly of type I collagen and is very similar to the acid denatured porcine skin type I collagen that is immobilized onto dextran beads to produce Cytodex 3 microcarriers. The remaining cell adhesion proteins used were undenatured and consisted of human placental type IV collagen (from Sigma), and human fibronectin.

Three reagents were used to increase the positive charge density on microcarrier beads. Polylysine (poly-DL-lysine, MW 15,000 to 30,000 from Sigma) and chitosan (86% deacetylation, 0.8% w/v in 0.5% acetic acid having a viscosity of 1200 centiposes; obtained from Maripol Systems Inc., Excelsior, MN) are large polymers with high densities of primary amines. Dimethylaminopropylamine (DMAPA; from Aldrich) was immobilized via the primary amine so that a tertiary amine remained to provide a positive charge at physiologic pH. This tertiary amine is similar to that provided by the N,N-diethylaminoethyl (DEAE) groups on Cytodex 1 beads.

A heterobifunctional crosslinking agent (BBA-EAC-NOS; benzoylbenzoic acid - epsilonaminocaproic acid - N-oxysuccinimide) was synthesized and used to immobilize cell adhesion proteins and positively charged molecules onto polystyrene beads. Upon photoactivation, the BBA (benzoylbenzoic acid) moiety forms a highly reactive intermediate that couples to the carbon-hydrogen bonds of carbon-based polymers.

The EAC (epsilon amino caproic acid) provides a 6-carbon spacer between the photogroup and the biomolecule. Finally, the NOS moiety (N-oxysuccinimide) provides a functional group for crosslinking to primary amines on biomolecules.

BBA-EAC-NOS was synthesized by standard reaction procedures. BBA was converted to the acylchloride with oxalylchloride and reacted from toluene with EACA in aqueous NaOH with vigorous stirring. The resulting BBA-EACA was extracted with ethylacetate and reacted with N-hydroxysuccinimide through activation with dicyclohexylcarbodiimide to produce the active ester. The crude BBA-EAC-NOS was recrystallized from hot ethanol and stored dry.

The starting materials are available commercially. Benzoylbenzoic acid is available from Aldrich Chemical Company (Milwaukee, WI). Other reagents were of the highest available purity and were procured as needed from chemical supply companies.

Example 2: Immobilization of Cell Adhesion Factors and Positively-Charged Molecules onto Microcarrier Beads.

Preparation of Tritiated Cell Adhesion Proteins. Tritiated tracers of each cell adhesion protein were prepared and used to quantitate protein immobilization. The amines of gelatin, type IV collagen, and fibronectin were tritiated by methylation with formaldehyde, followed by reduction with $[^3H]NaBH_4$. The tritiated protein derivatives were separated from excess radiolabel by exhaustive dialysis. The specific activities of the radiolabeled proteins were determined by UV spectroscopy/microbiuret protein assay and liquid scintillation spectrometry.

Immobilization of Cell Adhesion Factors and Positively- Charged Molecules onto Polystyrene Beads. The heterobifunctional crosslinking agent, BBA-EAC-NOS, was used to covalently immobilize each of the cell adhesion proteins (gelatin, type IV collagen, fibronectin) and two of the positively charged molecules (polylysine and DMAPA) onto polystyrene beads. The BBA-EAC-NOS was added to the polystyrene beads and allowed to adsorb. Next, the proteins and/or positively charged molecules were added and allowed to react with the NOS moiety to produce covalent coupling to the spacer. Then the beads were photoactivated (at 320 nm) to covalently immobilize the spacer (and covalently coupled cell adhesion proteins and/or positively charged molecules) to the

polystyrene beads. Finally, loosely adherent proteins and positively-charged molecules were removed by overnight washing with the mild detergent Tween 20 in phosphate buffered saline (pH 7.2).

Immobilization of Positively Charged Molecules on Cytodex 3 Beads. Polylysine, chitosan, and DMAPA were individually immobilized onto Cytodex 3 beads. Chitosan was dialyzed exhaustively against pH 4 deionized water before use. Cytodex 3 beads and each of the amine sources were mixed in 0.25 M MES (2[N-morpholino]ethanesulfonic acid) buffer at pH 5. EDC (1-ethyl-3-[dimethylaminopropyl]carbodiimide) powder was added in several aliquots at 15 minute intervals to the final concentration of 0.1 M. The bead suspensions were agitated during the reaction. Finally, the beads were washed with Tween 20 in PBS to remove loosely adherent molecules.

Example 3: Cell Culture.

Cell Types and Culture Conditions. Green monkey kidney (Vero) and Chinese hamster ovary (CHO) cells were purchased from ATCC. Both cell types were passaged and maintained in 75 cm^2 flasks at 37°C in a 5% CO_2 environment. The Vero cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) and the CHO cells in the Ham's F-12 Nutrient Mixture. Each medium was supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 20 mM HEPES buffer, 0.2 mM sodium pyruvate, 100 ug/ml streptomycin, and 100 units/ml penicillin (final pH 7.1).

In order to standardize the physiology of cells prior to each experiment, cells were passed into 150 cm^2 flasks 2 to 3 days prior to inoculation of microcarrier beads Cells were trypsinized (0.05% trypsin, 0.53 mM EDTA in PBS) for removal from the flasks. For the microcarrier experiments, the cells

were centrifuged to remove the trypsin medium and resuspended to about 1 x 10^6 cells/ml in culture medium. The viable cell concentration was determined by Trypan dye exclusion (0.4% Trypan blue in 0.9% saline).

Initial Screening for Cell Attachment in 20 ml Liquid Scintillation Vials. A small scale assay was used to initially assay cell attachment to each modified microcarrier bead type. By allowing small quantities of beads and cells to be used, this assay allowed more types of bead coatings to be tested than would be possible if all assays were conducted in the larger spinner flask assays.

For the small scale cell attachment assay, 2 mg/ml of Cytodex 1 (bearing positively-charged moieties) and Cytodex 3 (bearing collagen) beads or 20 mg/ml of coated polystyrene beads were used. These respective bead concentrations produced the same volumes of beads per ml of media. After swelling and equilibration of each bead type in cell culture media, the respective volumes occupied per mg dry weight of packed Cytodex 1, Cytodex 3, or polystyrene beads were 20 ul, 17 ul, and 1.7 ul. For these cell attachment assays the volumes of packed beads used per ml of media were 34 ul beads/ml for Cytodex 3 and polystyrene beads and 40 ul beads/ml for Cytodex 1 beads.

The assays were conducted in siliconized 20 ml glass liquid scintillation vials. The beads (34 or 40 ul/ml) and cells (1.5 x 10^5 cells/ml) were added to a total volume of 15 ml per vial. Then the beads were kept in suspension by continuously rotating the vials with a motion that prevented the beads from settling and rubbing against each other. To achieve this motion, the vials were attached at a 45 degree angle

to a Labquake vial rotator (Labindustries Model No. 400-110) and rotated at 8 rpm. At timed intervals, the vials were removed, the beads were allowed to settle for about 30 seconds, and aliquots of the cells remaining in suspension were removed and counted. For the cell counts, the cells were stained by mixing with an equal volume of crystal violet (0.1% w/w) in 0.1 M citric acid, and then counted with a hemocytometer. Cell depletion from the medium was used as an indicator of cells attached to beads.

To verify that cells removed from the medium were indeed attached to microcarriers (and not lysed), cells attached to microcarriers were quantitated at the end of each cell attachment assay. One ml aliquots of well-agitated carrier medium were removed, the microcarriers were allowed to settle, and the settled microcarriers were resuspended in crystal violet citric acid as described above. After incubating 1 hour at 37°C, the suspension was sheared by sucking into and out of a Pasteur pipet to release nuclei, which were quantitated with a hemocytometer.

Cell Culture and Assays in Spinner Flasks. Microcarriers were cultured using previously described protocols with 100 ml cultures being maintained in 250 ml spinner vessels and stirred with suspended magnetic impellers (50 rpm). The concentrations of beads and cells were the same as for the scintillation vial cell attachment assays. The bead concentrations were 2 mg/ml for Cytodex 1 and 3 and 20 mg/ml for polystyrene beads, and the cell concentration was 1.5×10^5 cells/ml for each cell type. The kinetics of cell attachment were assayed as a decrease in supernatant cell concentration using a protocol similar to that used for the scintillation vial cell attachment assays. For sample removal, the agitation was stopped

briefly (about 30 seconds) at which time the microcarriers settled and a supernatant sample was removed for cell quantitation as described above.

Starting at 4 hours, cells attached to microcarriers were assayed using the protocol described for quantitating cells attached to microcarriers at the end of the scintillation vial assays. All cultures were continued and assayed at daily intervals through 5 days, and at 2 to 3 day intervals thereafter. At 2 to 3 day intervals, the beads were allowed to settle and 50-75% of the culture medium was replaced with fresh medium.

The morphology of the cells growing on microcarriers was also assayed at timed intervals. Briefly, 0.1 ml aliquots of well-agitated carrier medium was removed and placed into individual wells of a 24-well cell culture plate. Then one drop (25 ul) of 0.5% crystal violet in 40% ethanol was added and incubated 30 seconds to fix and stain the cells. Then 3.0 ml of PBS was added to each well, and the cells were observed by bright field microscopy at 200X magnification.

Example 4: Quantitation of Tritiated Proteins Immobilized onto Polystyrene Beads.

The heterobifunctional crosslinking agent (BBA-EAC-NOS) was used to covalently immobilize cell adhesion proteins onto polystyrene beads using the protocol described above (Example 2). An initial experiment was conducted with [^{3}H]gelatin to determine: 1) the efficiency of protein binding and the maximum protein loading that could be achieved using the covalent immobilization protocol; and 2) how covalent loading compares to adsorption in the absence of BBA-EAC-NOS. Results shown in Table I show that loading of covalently bound gelatin saturated at about

3.5 ug per 10 ul of beads. The efficiency of gelatin loading was 93% when added at 3 ug gelatin per 10 ul beads; whereas at the higher levels of added gelatin, the percentage of binding decreased with little increase in total loading. These results indicate that 3 ug gelatin added per 10 ul beads would couple quite efficiently and yield nearly maximal loading. This ratio of gelatin added to beads was used for the remainder of the study.

TABLE I. CELL ADHESION PROTEINS IMMOBILIZED ONTO POLYSTYRENE BEADS.

Protein	Covalent or Adsorbed	Protein added /10 ul beads (ug)	Protein Bound /10 ul beads (ug)	% Immob.	Fold Increase (C/A)
Gelatin	C	3.0	2.8	93	4.8
Gelatin	A	3.0	0.58	19	---
Gelatin	C	7.3	3.3	45	4.6
Gelatin	A	7.3	0.70	9.6	---
Gelatin	C	18.0	3.4	19	4.3
Gelatin	A	18.0	0.80	4.4	---
IV Collagen	C	0.96	1.11	116	7.9
IV Collagen	A	0.96	0.14	14	---
Fibronectin	C	1.22	1.08	89	3.2
Fibronectin	A	1.22	0.34	28	---

When covalent immobilization of gelatin is compared to adsorption (at 3-18 ug added per 10 ul beads), it is seen that 4-to 5-fold more protein is immobilized via the BBA-EAC-NOS than is immobilized by adsorption. Results presented below (Example 8) demonstrate that cells bind faster and more tenaciously to covalently immobilized cell adhesion proteins and positively charged moieties than to the same reagents when they are adsorbed without covalent bonding.

Tritiated type IV collagen and fibronectin exhibited similar binding characteristics to those observed with gelatin (Table I). Each of these proteins was added at a concentration of about 1 ug per 10 ul beads and covalently coupled with an efficiency of 89% or higher. Also, each protein produced several fold more protein covalently immobilized than adsorbed. For the remaining experiments, 3 ug of gelatin or 1 ug type IV collagen or fibronectin were added per 10 ul polystyrene beads.

These results clearly demonstrate that this covalent coupling technology utilizing BBA-EAC-NOS produces both efficient binding of proteins to polystyrene beads, and significantly increases the protein loading density when compared to adsorption.

Example 5: Calculation of Cell Attachment Rate Constants.

Cytodex 1 microcarriers have a high density of positive charges and are typically reported to produce faster cell attachment than do Cytodex 3 microcarriers. In Table II, the initial rate of cell attachment to each microcarrier type is expressed as a rate constant, which was calculated from the formula:

$$K(min^{-1}) = \frac{\ln [\text{initial cell no.}] - \ln [\text{final cell no.}]}{\text{min.}}$$

as previously described (Himes, V.B. and W-S. Hu, 1987, Biotech. Bioeng. 24:1155-1193). Table II shows that: 1) Vero cells attach about twice as fast to Cytodex 1 microcarriers as to Cytodex 3 microcarriers, and 2) both cell attachment assays produced nearly identical results.

As was described in Example 2, the cells attached at each assay time were determined by quantitating the removal of cells from the culture medium. However, to verify that the removed cells had

bound to microcarriers (and had not lysed), the cells attached to microcarriers were also quantitated at the end of each assay (at 60 minutes). Table II shows that 92-100% of the cells were attached to the beads at 60 minutes, therefore verifying that cell depletion from the medium was due to attachment to the microcarriers.

TABLE II. RATE CONSTANTS FOR ATTACHMENT OF VERO CELLS TO CYTODEX 1 AND CYTODEX 3 MICROCARRIERS ASSAYED IN EITHER THE SCINTILLATION VIAL ASSAY OR THE SPINNER FLASK ASSAY.

Assay Protocol	Microcarrier Type	Rate Constant (min^{-1})	% of Cells Attached at 60 min.
Vial	Cytodex 1	0.212	97
Vial	Cytodex 3	0.115	92
Flask	Cytodex 1	0.226	100
Flask	Cytodex 3	0.129	99

Example 6: Cell Attachment to Cytodex 3 Microcarriers with Added Positively Charged Groups.

To determine whether increasing the density of positive charges would increase the rate of cell attachment to Cytodex 3 microcarriers (having collagen on surface), three different molecules were added (using protocols described in Example 2) to introduce positively charged groups. Chitosan and polylysine each added primary amines, whereas the DMAPA added tertiary amines similar to the DEAE present on Cytodex 1.

Table III shows that each of the molecules used to add positive charges to Cytodex 3 microcarriers increased the cell attachment rates of both Vero and CHO cells. Chitosan was the most effective (see Table III for CHO cells and compare Tables II and III for Vero cells).

TABLE III. RATE CONSTANTS FOR ATTACHMENT OF CELLS TO CYTODEX 3 MICROCARRIERS MODIFIED BY THE ADDITION OF POSITIVELY CHARGED GROUPS.

Microcarrier Type	Cell Type	Rate Constant (min^{-1})	% of Cells Attached at 60 min.
Cytodex 3	Vero	0.164	96
Cytodex 3 + chitosan	Vero	0.271	93
Cytodex 3 + polylysine	Vero	0.237	94
Cytodex 3 + DMAPA	Vero	0.204	96
Cytodex 3	CHO	0.037	80
Cytodex 3 + chitosan	CHO	0.085	82
Cytodex 3 + polylysine	CHO	0.052	82

Example 7: Cell Attachment to Polystyrene Beads with Individually Added Cell Adhesion Proteins or Positively Charged Molecules.

The positively charged molecules shown above (Table III) to improve cell attachment to Cytodex 3 microcarriers were individually immobilized onto polystyrene beads (using protocols described in Example 2) and assayed for cell attachment using Vero cells. For each set of cell attachment experiments, Cytodex 3 microcarriers were included as a control to verify that the batch of cells exhibited normal attachment kinetics. Results shown in Table IV reveal: 1) no cells attached to uncoated polystyrene (polystyrene-uncoated); and 2) the polylysine coating produced cell attachment comparable to that observed with Cytodex 3 microcarriers.

TABLE IV. RATE CONSTANTS FOR ATTACHMENT OF CELLS TO POLYSTYRENE MICROCARRIERS WITH INDIVIDUALLY ADDED POSITIVELY CHARGED REAGENTS.

Microcarrier Type	Cell Type	Rate Constant (min^{-1})	% of Cells Attached at 60 min.
Cytodex 3	Vero	0.163	97
Polystyrene-uncoated	Vero	0.000	0
Polystyrene + polylysine	Vero	0.169	90

Cell adhesion proteins were individually immobilized onto polystyrene microcarriers (using protocols described in Example 2) and assayed for cell attachment using either Vero or CHO cells. Table V shows that none of the cell adhesion proteins immobilized onto polystyrene beads produced cell attachment equivalent to Cytodex 3 microcarriers. With the coated polystyrene microcarriers, cell attachment was fastest with type IV collagen, next fastest with gelatin, and slowest with fibronectin.

TABLE V. RATE CONSTANTS FOR ATTACHMENT OF CELLS TO POLYSTYRENE MICROCARRIERS WITH INDIVIDUALLY ADDED CELL ADHESION PROTEINS.

Experiment Number	Microcarrier Type	Cell Type	Rate Constant (min^{-1})	% of Cells Attached at 60 min.
1	Cytodex 3	CHO	0.043	76
1	Polystyrene + IV collagen	CHO	0.034	[illegible]
1	Polystyrene + fibronectin	CHO	0.016	27
2	Cytodex 3	Vero	0.171	94
2	Polystyrene + IV collagen	Vero	0.075	63
2	Polystyrene-uncoated	Vero	0.000	0
3	Cytodex 3	Vero	0.136	97
3	Polystyrene + gelatin	Vero	0.089	49

4	Cytodex 3	Vero	0.190	97
4	Polystyrene + fibronectin	Vero	0.012	21

[a] The beads coated with type IV collagen showed maximum binding at 20 minutes (49%), after which steadily decreasing percentages of attached cells were measured until no attached cells were observed at 60 minutes.

Example 8: Cell attachment to polystyrene beads coated with polylysine plus either type IV collagen or gelatin.

When polylysine was added to polystyrene microcarriers, it produced cell attachment comparable to Cytodex 3 (Table IV); and of the three cell adhesion proteins tested, type IV collagen and gelatin produced the fastest cell attachment (Table V).

Polystyrene microcarriers were prepared that had immobilized either: 1) polylysine plus type IV collagen, or 2) polylysine plus gelatin. Each reagent combination was immobilized by addition to BBA-EAC-NOS treated polystyrene beads as described in Example 2. Since the polylysine and each protein compete for binding to NOS moieties, the immobilized polylysine decreases the loading level of each protein. The ratios of each protein and polylysine were adjusted so that each protein was immobilized at about 70% of the levels present on the microcarriers used in Table V.

Table VI shows that a combination of polylysine plus either type IV collagen or gelatin added to polystyrene microcarriers produced attachment of each cell type that was equal to or better than attachment to Cytodex 3 microcarriers. Adsorption controls for which the same concentrations of gelatin and polylysine were added in the absence of the crosslinking agent, BBA-EAC-NOS, produced: 1) 50% to 70% decreases in the initial cell attachment rates for each cell type, and 2) an apparent release of Vero cells after initial

attachment. This demonstrates the essential contribution of the covalent crosslinking agent.

TABLE VI. RATE CONSTANTS FOR ATTACHMENT OF CELLS TO POLYSTYRENE MICROCARRIERS COATED WITH POLYLYSINE PLUS EITHER TYPE IV COLLAGEN OR GELATIN.

Experiment Number	Microcarrier Type	Cell Type	Rate Constant (min^{-1})	% of Cells Attached at 60 min.
1	Cytodex 3	Vero	0.162	78
1	PS + PL + IV collagen	Vero	0.236	92
1	PS + PL + gelatin	Vero	0.193	98
1	PS + PL + gelatin (ads.)	Vero	0.104	42[a]
2	Cytodex 3	CHO	0.040	71
2	PS + PL + gelatin	CHO	0.043	82
2	PS + PL + gelatin (ads.)	CHO	0.012	27

[a] The beads coated with adsorbed reagents showed maximum binding (81%) at 15 minutes, followed by steadily decreasing percentages of cells attached through 60 minutes.

PS = polystyrene
PL = polylysine
ads. = reagents adsorbed to polystyrene beads in the absence of BBA-EAC-NOS

Example 9: Spinner Flask Assays.

Results from the scintillation vial cell attachment assays indicate that: 1) charged groups added to Cytodex 3 microcarriers greatly improved the rate of cell attachment (Table III); and 2) polystyrene beads modified by covalent immobilization of polylysine plus either gelatin or type IV collagen produced cell attachment equal to or better than Cytodex 3 microcarriers (Table VI).

Cell Attachment. Cell attachment was assayed in spinner flasks using the protocol described in Example

3. Table VII, Experiment 1 compares Vero cell attachment with four microcarrier types: Cytodex 1, Cytodex 3, Cytodex 3 + chitosan, and polystyrene (PS) + polylysine (PL) + gelatin. The two microcarrier types coated with both gelatin and a positively charged moiety produced the fastest rates of initial cell attachment, and all four microcarrier types produced 95-100% cell attachment by 60 minutes.

TABLE VII. RATE CONSTANTS FOR ATTACHMENT OF CELLS TO MICROCARRIERS ASSAYED IN SPINNER FLASKS.

Experiment Number	Microcarrier Type	Cell Type	Rate Constant (min^{-1})	% of Cells Attached at 60 min.
1	Cytodex 1	Vero	0.226	100
1	Cytodex 3	Vero	0.129	99
1	Cytodex 3 + chitosan	Vero	0.290	100
1	PS + PL + gelatin	Vero	0.260	95
2	Cytodex 1	CHO	0.112	99
2	Cytodex 3	CHO	0.075	96
2	Cytodex 3 + chitosan	CHO	0.134	98

PS = polystyrene PL = polylysine

Table VII, Experiment 2 compares CHO cell attachment to several microcarrier types. Cytodex 1 and Cytodex 3 + chitosan showed higher rates of cell attachment than did Cytodex 3.

These results agree with the scintillation vial results by confirming that chitosan greatly improves the attachment of both cell types to Cytodex 3 microcarriers.

Cell Growth. Cells attached to microcarriers were assayed initially at 4 hours and later at daily intervals, as describedin Example 3. Cell numbers per ml of culture medium are given for days 2 and 8 in Table VIII.

TABLE VIII. CELL GROWTH ON MICROCARRIERS ASSAYED IN SPINNER FLASKS.

Microcarrier Type	Cell Type	cells per ml (x 10^6) Day 2	Day 8
Cytodex 1	Vero	1.3	4.4
Cytodex 3	Vero	1.6	4.2
Cytodex 3 + chitosan	Vero	2.2	7.7
PS + PL + gelatin	Vero	2.0	6.4
Cytodex 1	CHO	1.3	2.8
Cytodex 3	CHO	1.6	3.2
Cytodex 3 + chitosan	CHO	1.8	3.3

The Vero cells grew best on the two microcarrier types coated with gelatin plus positively charged groups: polystyrene microcarriers coated with polylysine plus gelatin (PS + PL + gelatin) and Cytodex 3 microcarriers coated with chitosan. The CHO cells grew best on Cytodex 3 and Cytodex 3 plus chitosan.

These results clearly show that adding positively charged groups to gelatin does not decrease cell growth, since the Cytodex 3 plus chitosan produced excellent growth of both cell types and polystyrene coated with gelatin plus polylysine produced excellent Vero cell growth.

While a preferred embodiment of the present invention has been described, it should be understood that various changes, adaptations and modifications may be made therein without departing from the spirit of the invention and the scope of the appended claims.

WHAT IS CLAIMED IS:

1. A cell culture system comprising a supporting surface for attachment of cells, the surface bearing a composition comprising a positively-charged moiety and a cell adhesion factor.

2. The system of claim 1 wherein the positively-charged moiety and the cell adhesion factor each are bound to said surface.

3. The cell culture system of claim 1 in which the cell adhesion factor and the positively-charged moiety are bound to one another and either the cell adhesion factor or the positively-charged moiety is bound to the supporting surface.

4. The cell culture system of claim 1 wherein said cell adhesion factor is selected from the group consisting of fibronectin, laminin, collagens (all types), vitronectin, and tenascin.

5. The cell culture system of claim 1 wherein the cell adhesion factor is a peptide comprising the attachment domain of a naturally occurring protein having not more than about 30 amino acid moieties in its amino acid sequence.

6. The cell culture system of claim 1 wherein the positively-charged moiety comprises an amine.

7. The cell culture system of claim 1 wherein the mole ratio of the positively charged moiety to the cell adhesion factor is in the range of about 10:1 to about 1^6:1.

8. The cell culture system of claim 1 wherein the density of the positively charged moiety upon the supporting surface is sufficient to promote cell attachment thereto.

9. The cell culture system of claim 8 wherein the density of the positively charged moiety upon the

supporting surface is in the range of about 0.02 to about 2.0 meq/g.

10. The cell culture system of claim 1 wherein the cell culture system is a microcarrier.

11. The cell culture system of claim 1 wherein the density of cell adhesion factor upon the supporting surface is sufficient to stablilize attachment of such cells to the surface.

12. The cell culture system of claim 11 wherein the density of cell adhesion factor upon the supporting surface ranges from about 0.1 nanomole to about 10 micromoles of factor per gram.

13. The cell culture system of claim 1 in which at least one of the cell adhesion factor and the positively charged moiety is covalently bonded to the surface of the supporting surface.

14. The cell culture system of claim 1 including linking groups covalently linking either or both of the cell adhesion factor and the positively charged moiety to said surface, the linking groups including the residue of a latent reactive group covalently bonded to the surface.

15. The cell culture system of claim 1 further comprising another cell adhesion factor carried upon the supporting surface.

16. A method of making a cell culture system comprising:

Attaching to a supporting surface of the system a composition comprising a positively-charged moiety and a cell adhesion factor.

17. The method of claim 16 wherein each of the positively-charged moieties and cell adhesion factors is attached to the surface through covalent bonds

18. The method of claim 16 further comprising the step of binding the cell adhesion factor to the positively-charged moiety before attaching them to the supporting surface via either the cell adhesion factor or the positively-charged moiety.

19. A method of growing anchorage-dependent cells on a supporting surface of a system comprising growing cells in an aqueous environment on the supporting surface bearing a composition comprising a positively-charged moiety and a cell adhesion factor.

20. The method of claim 19 wherein the density of the positively charged moiety attached to the supporting surface is sufficient to promote cell attachment thereto.

21. The method of claim 20 wherein the density of the positively charged moiety attached to the supporting surface is in the range of about 0.02 to about 2.0 meq/g.

22. The method of claim 16 wherein the cell culture system is a microcarrier.

23. The method of claim 22 wherein the density of cell adhesion factor attached to the supporting surface is sufficient to stablilize attachment of such cells to the surface.

24. The method of claim 23 wherein the density of cell adhesion factor attached to the supporting surface ranges from about 0.1 nanomole to about 10 micromoles of factor per gram of microcarrier.

25. The method of claim 16 further including the step of attaching another cell adhesion factor to the supporting surface